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Investigations of Aroma Volatile Biosynthesis under Anoxic Conditions and in Different Tissues of "Redchief Delicious" Apple Fruit (*Malus domestica* Borkh.)

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Disks from different tissues were obtained from "Redchief Delicious" apple fruit (*Malus domestica* Borkh.) and analyzed for the ability to metabolize 1-pentanol as well as synthesize constitutive esters and alcohols under anoxic and aerobic conditions. The skin tissue displayed a greater capacity to synthesize pentanal, pentyl acetate, pentyl propionate, pentyl butyrate, and pentyl hexanoate than the hypanthial and carpellary tissues during incubation with 1-pentanol. With the exception of pentyl acetate and pentyl propionate biosynthesis, the hypanthial tissue synthesized these compounds at a higher rate than the carpellary tissue. Anoxia inhibited both constituent and 1-pentanol-derived ester biosynthesis. While anoxia inhibited ester biosynthesis, ethanol biosynthesis increased at a greater rate in tissue disks held under these conditions. Biosynthesis of 1-butanol, 2-methyl-1-butanol, and 1-hexanol was greater in tissue disks held in air during the first part of the measurement period and dropped off more rapidly than those transpiring in tissue disks held under anoxic conditions. The biosynthetic rates of all esters, both constituent and 1-pentanol-derived, increased as a result of air exposure. While hypoxic or anoxic conditions may promote ethanol synthesis, these conditions also appear to inhibit the formation of the ethanol-derived esters partially responsible for the off-flavor in apples attributed to ultralow O_2 controlled atmosphere storage.

KEYWORDS: ***Malus domestica*; apple; aroma; controlled atmosphere; anatomy; anoxia**

INTRODUCTION

Ester biosynthesis is a key component of apple fruit aroma generation (*1*). Biosyntheses of principle aroma esters of apple fruits are the product of the metabolism of fatty acids (*2*, *3*) and specific amino acids (*4*, *5*). Fatty substrates for the apple fruit aroma volatile pathway mainly result from the activity of lipoxygenase (*6*), β-oxidation (*2*), and α-oxidation (*3*). Oxidized fatty acids may undergo reduction to an aldehyde or primary alcohol or esterification with coenzyme A (CoA) (*2*, *7*). The reaction catalyzed by alcohol acetyltranferase (AAT) results in the esterification of the primary alcohol and a fatty acyl CoA (*8*). Apple fruit ester hydrolase (esterase) may catalyze the hydrolysis of these ester bonds, indirectly forming primary alcohols and a fatty acyl CoA (*9*). Furthermore, primary alcohols may be oxidized to form aldehydes and fatty acids (*3*, *10*).

Apple fruit aroma is thought to be synthesized largely in the skin tissue (*7*, *11*). The volatile synthesis capacity of the carpellary tissue was not investigated by these studies. Leshem et al. (*12*) reported higher lipoxygenase activity in the carpellary tissue (between the carpels and the core line) than the hypanthial tissue (between the skin and the core line) of poststorage apple fruit. This evidence, along with high levels of ethylene production in the carpellary tissue when compared to the hypanthial tissue of both pre- and postclimacteric "Fuji" apple fruit (*13*), and evidence of the ethylene dependence of certain ester biosyntheses (*14*) suggest that further investigation of the volatile biosynthetic capacity of apple carpellary tissue is needed.

Generally, anoxia and/or hypoxia inhibit the formation of key aroma compounds (*15*). The inhibition is more pronounced with increasing length of storage under hypoxic conditions, decreasing storage temperature (*15*), decreasing atmospheric oxygen content during storage (*16*), and decreasing maturity at harvest (*17*). High atmospheric CO_2 concentrations adversely affect straight chain aroma volatile synthesis during storage (*18*). Ethanol production is stimulated under hypoxic storage conditions (*19*, *20*). In organs of higher plants kept under anoxic

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conditions, ethanol arises largely from the fermentation pathway (*21*). Ethanol accumulation during storage leads to the disproportionate synthesis of ethanol-derived esters (*22*). Because of their low organoleptic thresholds (*23*), disproportionate amounts of these volatiles may be responsible for some of the off-flavors attributed to fruit stored in hypoxic controlled atmosphere (CA) conditions.

The present study investigates the differences in metabolism of 1-pentanol between different apple fruit tissues and the effects of anoxia on the biosyntheses of constituent and feed alcohol-derived aroma compounds of poststorage apple fruit using solid phase microextraction (SPME)/capillary gas chromatography.

MATERIALS AND METHODS

Redchief Delicious apple fruits were harvested during the 1998 growing season from the Tukey Research orchard, Pullman, WA, and stored at 0 °C in air. The investigations of anaerobic and aerobic volatile syntheses were performed using apple fruits sampled at 124 days after full bloom (DAFB) and subsequently stored for approximately 5 months. The investigation of the volatile synthesis capabilities of the different apple fruit tissues was performed using apples harvested at 151 DAFB and stored for approximately 5 1/2 months. After they were removed from storage, fruits were allowed to acclimate for 48 h at 21 °C.

Fruit used for the investigation of anaerobic and aerobic volatile synthesis were cut into transverse sections, approximately 3 mm wide, starting at the equator. Subsequently, tissue disks were excised from the slices using a 9 mm (diameter) stainless steel cork borer. The disks were promptly weighed and placed in 25 mL Erlenmeyer flasks. Samples contained approximately 3 g of tissue from an equal mix of four different apple fruit. The flasks were then sealed using rubber serum stoppers. Flasks were placed in a flow of either N_2 gas or compressed air. The gases passed through a cartridge containing a molecular sieve, $CaSO_4$, and deionized water (to maintain humidity) before entering the sample flask. At exactly 60 min following the sealing of the flasks, some samples were treated by adding 5 μL of 1-pentanol (Aldrich, Milwaukee, WI) to the flask. The flows of the treatment gases were adjusted to 3.4 mL min^{-1}. Oxygen concentrations of the samples were monitored using a Systech Instruments (Chicago, IL) model 2550 O_2 monitor. Complete pull-down (to <0.001% O_2) times for the N_2-treated samples were approximately 10 min.

Disks used for volatile production comparison of different tissues were prepared by peeling the skin of the fruit, just under the hypodermis, in large sheets. The remainder of the fruit was cut into 3 mm wide slices as previously described. Disks were removed from the skin tissue, the hypanthial tissue, and the carpellary tissue using a 9 mm (diameter) cork borer. Tissue disks were placed in 25 mL Erlenmeyer flasks, treated with 5 μL of 1-pentanol (Aldrich) after 15 min, and sealed with a serum stopper. Each flask (3 flasks/treatment) contained a bulk tissue sample from four different fruits. The headspace of these samples remained static (no flow).

Headspace volatile analyses were performed by exposing a SPME fiber (Supelco Co., Bellefonte, PA) coated with a 100 μm thick layer of poly(dimethylsiloxane) to the headspace. The SPME device was inserted into the flask through the serum stopper, and the fiber extended from its protective sheath to expose the sorption surface for a period of 15 min. The adsorbed contents of the SPME fiber were analyzed by exposing the fiber and desorbing the sample into the injection port of a Hewlett-Packard (HP, Avondale, PA), 5890 gas chromatograph equipped with a DB-1 (J&W, Folsom, CA) fused silica capillary column (60 m × 0.32 mm i.d. × 0.25 μm film thickness), and a HP 5970 mass selective detector. The injector and transfer line temperatures were maintained at 200 and 250 °C, respectively. The injector remained in the splitless mode for the first 2 min of the injection. The column oven temperature was held at 35 °C for 3 min and then increased at a rate of 8 °C min^{-1} to 225 °C. Data were collected and analyzed using the HP Chemstation G 1034C data processing package. Matching spectra to those cataloged in the Wiley/NBS library tentatively identified compounds of interest. Comparing the unknown retention time and spectra to those of authentic standards confirmed tentative identifications. Quantification was accomplished using response factors obtained by exposing the SPME fiber to a headspace containing a known concentration of each standard for 15 min and desorbing the fiber as previously described. Data from the tissue-based analyses were averaged and reported with the standard error (SE) of the means (df = $n - 1$). Data from each of the samples from the anoxia analyses were plotted alone in sequence.

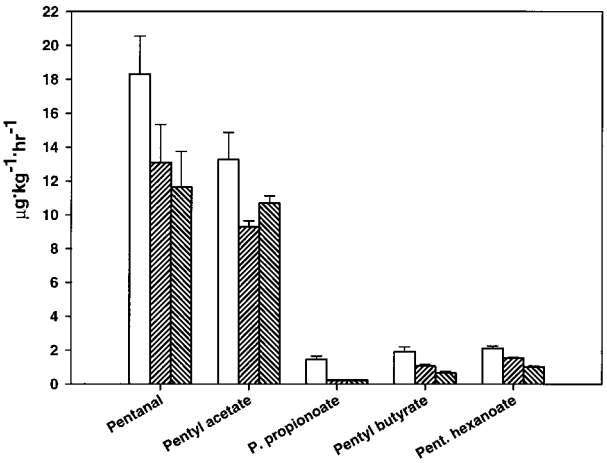

Figure 1. Biosynthesis of 1-pentanol-derived aroma volatiles by different apple fruit tissue types (□, skin; ▨, hypanthium; ▧, carpellary). Vertical bars represent SE ($n = 3$); where no bars appear, SE was smaller than the bar size.

RESULTS AND DISCUSSION

Formation of Volatile Esters Derived from 1-Pentanol. The in situ biosynthesis of 1-pentanol in apple fruit reportedly occurs through the α-oxidation of hexanoate (an oxidized lipoxygenase system product) to pentanoate and its subsequent reduction to 1-pentanol (*3*). In every tissue type, many volatile constituents including pentanal, pentyl acetate, pentyl propionate, pentyl butyrate, pentyl pentanoate, and pentyl hexanoate were produced during exposure to 1-pentanol (**Figure 1**). None of these compounds were detected in untreated fruit with the exception of traces of pentyl acetate. The synthesis of pentanal results from the oxidation of 1-pentanol (*3*, *24*). Pentyl acetate, pentyl butyrate, and pentyl hexanoate are synthesized from 1-pentanol (the alcohol moiety) and the specific constituent acid moiety (*3*, *7*). The esterification of 1-pentanol and the product of its oxidation, pentanoyl−CoA, result in the formation of pentyl pentanoate. Similarly, the synthesis of pentyl propionoate most likely results from the esterification of 1-pentanol with its oxidized product (to pentanoyl−CoA and through 1 cycle of β-oxidation). The oxidation of a feed alcohol by apple fruit tissue and its subsequent β-oxidation have been previously reported (*3*, *10*, *24*).

Volatile Production of Different Apple Fruit Tissues after Storage. Aroma volatile production in apple fruit occurs to a higher degree in the skin than the hypanthial tissue of poststorage apple fruit but has not been investigated in the carpellary tissue (*7, 11*). Overall, in the present investigation, metabolism of 1-pentanol was higher in the skin while decreasing toward the center of the fruit (**Figure 1**). However, pentyl acetate and pentyl propionate production were higher or similar among the hypanthial and carpellary tissues. Pentanal and pentyl acetate production were synthesized at a higher rate in all tissues than the other reported compounds detected.

As production of pentyl acetate, pentyl butyrate, and pentyl hexanoate appears to be largely dependent on the presence of these specific acid moieties under these test conditions, a higher biosynthetic rate of a pentyl ester corresponding to a specific acid may indicate a greater biosynthetic rate of that acid moiety and/or greater esterification activity in these different tissues. Similarities in the ratios of pentyl butyrate to pentyl hexanoate biosyntheses among different tissue types, as well as biosynthetic rate, may indicate that these acid moieties are of similar origin. The production of each of these acid moieties may be dependent upon the activity of lipoxygenase, producing hexanal (*6*), which could be oxidized to acetic and/or butyric acid via β-oxidation (*2*). Also, it is possible that the disk preparation may have stimulated greater lipoxygenase activity and production of hexanal (*6*).

Leshem et al. (*12*) reported that lipoxygenase activity is higher in the carpellary than hypanthial tissue of poststorage apple fruit. Our results indicate that the skin, followed by the hypanthial and then the carpellary tissue, has a significantly greater ability to produce these fatty acid precursors in poststorage fruit. The data suggest that lipoxygenase activity may be higher in the skin tissue followed by the hypanthial and carpellary tissue, respectively. However, the differences in availability of these precursors may result from a lack of substrate availability for the lipoxygenase, a different isoform of lipoxygenase, a difference in the entire lipoxygenase pathway whose catalytic activity may not result in the biosynthesis of these products (such as a higher amount of a non-C13 specific lipoxygenase in a certain tissue), or the use of fruit at a different stage of maturity than those used by Leshem et al. (*12*).

The differences in the biosynthetic rate of pentyl acetate in each of the tissues are less descriptive. The lack of similarity between the production of this ester, pentyl butyrate, and pentyl hexanoate suggests that some of the acetate may have originated from a different source than that of the latter two esters, particularly in the carpellary tissue, where the production of this ester does not occur in the same pattern as the pentyl butyrate and pentyl hexanoate.

The rates of pentanal and pentyl propionate production are dependent on the oxidation of the introduced 1-pentanol since these compounds are not produced in nontreated tissue disks. Oxidation of 1-pentanol to pentanal is somewhat higher in the skin tissue. Pentyl propionate production is substantially lower than that of pentanal. In all of the tissues, production of both of these metabolites is higher in the skin suggesting that oxidation, possibly β-oxidation, is greater in the peel tissue.

This evidence agrees with that presented in previous reports showing that volatile production is greater in the skin tissue when compared to the hypanthial tissue (*7*, *11*). In addition, it suggests that the skin tissue has a greater capacity to produce volatiles than the carpellary tissue. Furthermore, the differences in volatile production capacity may reflect reduced oxidative capacity in the hypanthial and accessory tissues in these poststorage apple fruits. The effect of lipoxygenase activity in the various tissues on volatile precursor synthesis by both pre- and poststorage fruit needs further investigation.

Effect of Anoxia on 1-Pentanol-Derived Aroma Volatiles. Treatment of disks with 1-pentanol leads to the biosyntheses of pentanal, pentyl acetate, pentyl propionate, pentyl pentanoate, ethyl pentanoate, pentyl butyrate, and pentyl hexanoate. The biosynthesis of pentyl butyrate and pentyl hexanoate was adversely affected by anoxic conditions (**Figure 2**). The biosynthesis of pentyl acetate sharply increased following a brief decrease that occurred at the beginning of the measurement

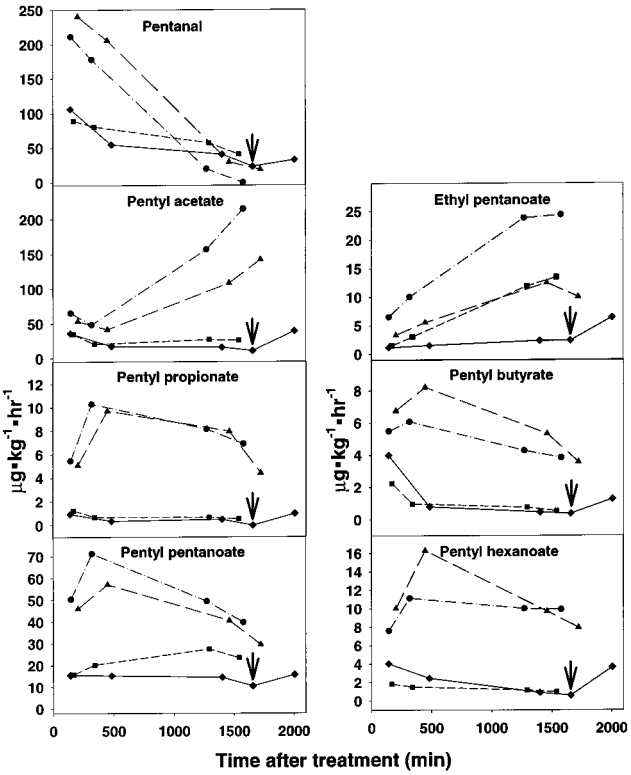

Figure 2. Biosynthesis of 1-pentanol-derived aroma volatiles by hypanthial tissue disks kept in air (●, ▲) or N_2 (■, ◆). Arrows designate the point at which a sample held under anoxic conditions was subsequently exposed to air. Each symbol in a treatment represents samples prepared from apples removed from storage at monthly intervals as described in the Materials and Methods.

period under aerobic conditions and remained stable under anoxic conditions. Pentanal, pentyl pentanoate, and pentyl propionate biosyntheses also decreased under anoxic conditions. Differences in biosynthesis of ethyl pentanoate were less obvious. This compound requires both the oxidation of added 1-pentanol and the biosynthesis of constituent ethanol. It appears as if the synthesis of this compound may have been dependent upon the fruits used during a certain test period. If the biosynthesis of ethyl pentanoate by the anoxic and aerobic treatments is compared by testing date, it appears that anoxia adversely affects its production. In every treatment, the biosyntheses of all pentanal-derived compounds increased once samples held under anoxic conditions were exposed to air.

Effects of Anoxia on the Production of Constituent Compounds. Ethanol biosynthesis increased in apple fruit tissue under both aerobic and anoxic conditions (**Figure 3**) and had the highest concentration of any compound detected. The increase in ethanol biosynthesis was greater in tissue disks exposed to anoxic conditions. Accumulation of ethanol in whole apple fruits held under hypoxic and anoxic conditions has been previously reported (*19*, *20*, *25*).

The biosynthetic rate of 1-butanol was highest followed by 2-methyl-1-butanol and then 1-hexanol (**Figure 4**). Subsequent exposure to air led to increased 1-butanol and decreased 1-hexanol production. 2-Methyl-1-butanol biosynthesis remained unchanged. This could be indicative of differences reported in the biosynthetic origins of 2-methyl-butanol in apple fruit (*5*) or rapid esterification upon exposure to air. The first measurements (at approximately 200 min after initiation of each treatment) of acetate esters containing these alcohol moieties were considerably higher in air than in the anoxic environment

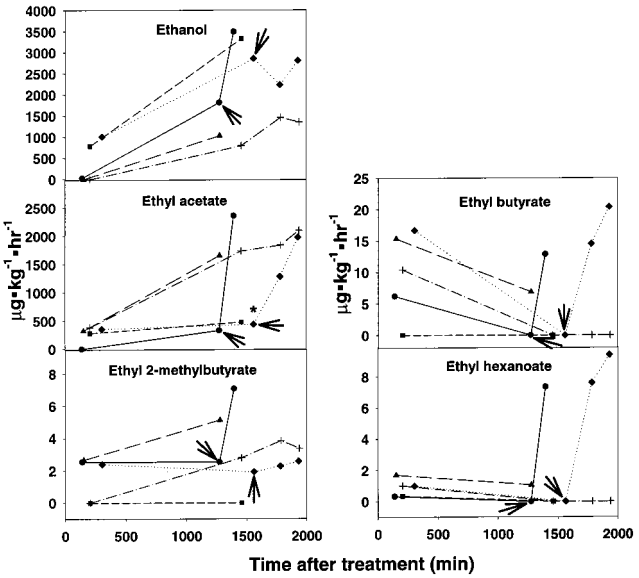

Figure 3. Biosynthesis of constituent ethanol and ethanol-derived esters by hypanthial tissue disks kept in air (▲, +) or N_2 (●, ■, ◆). Arrows designate the point at which samples held under anoxic conditions were subsequently exposed to air.

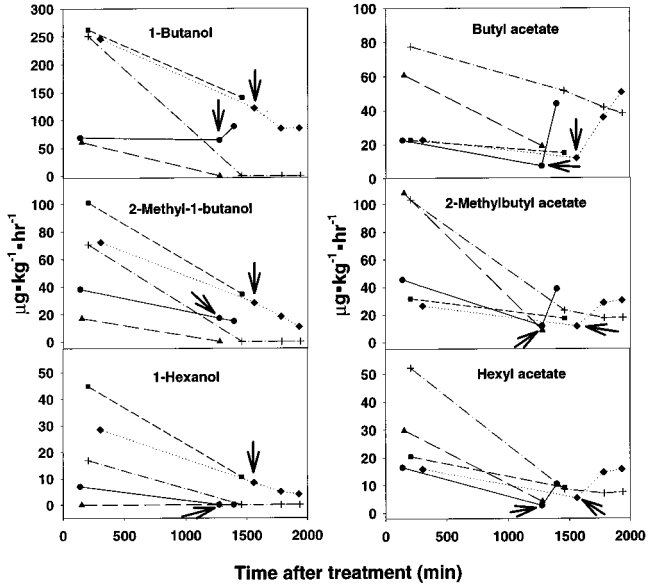

Figure 4. Biosynthesis of constituent alcohols and esters by hypanthial tissue disks kept in air (▲, +) or N_2 (●, ■, ◆). Arrows designate the point at which samples held under anoxic conditions were subsequently exposed to air.

(**Figure 4**). However, as with their specific alcohol precursors, the biosyntheses of these esters in air decreased more rapidly than in the anoxic environment. This may result from a gradual decrease in lipoxygenase activity or decrease in the availability of substrate as time accrued between tissue preparation and sampling coupled with the rapid esterification of these alcohols. Interestingly, the biosynthetic rates of 2-methyl-1-butanol and 2-methylbutyl acetate as well as 1-hexanol and hexyl acetate are similar. This is not the case with 1-butanol and butyl acetate, the latter of which has a lower production rate, suggesting that a larger amount of 1-butanol may be metabolized for the production of compounds other than its acetate ester. The rate of biosynthesis of these acetate esters in the anaerobic tissue disks all increased upon exposure to air.

Mattheis et al. (*22*) reported an increase in ethyl ester production, especially ethyl acetate, in whole apple fruit after hypoxic storage. Ethanol biosynthesis increased in the anoxic tissue disks in the present study as well as in the air-treated disks, albeit to a lesser extent. Ethyl acetate production increased steadily in the air-treated disks during the measurement period (**Figure 3**). However, ethyl acetate biosynthesis under anoxic conditions remained relatively stable and then increased rapidly upon exposure to air. Ethyl acetate production was approximately 100-fold higher than production of other ethyl esters and reflects the magnitude of ethanol production. This may reflect the abundance of ethanol for use as a substrate or possibly an affinity of AAT for ethanol and acetyl CoA and not larger acid moieties coupled with ethanol. However, substrate specificity studies of esterification of various alcohol moieties and acetyl CoA found that strawberry fruit AAT has a decreasing affinity for straight chain alcohol moieties smaller than 1-hexanol (*26*). Because ethyl acetate production increases followed a different timetable in melon, Wang et al. (*27*) suggested that its precursors may differ from other esters. Ethyl acetate production followed more closely to that of alanine in their study. Ethyl 2-methylbutyrate biosynthesis followed a pattern similar to that of ethyl acetate under the same conditions. Ethyl butyrate and ethyl hexanoate biosynthesis, like 1-butanol and 1-hexanol, decreased under both anoxic and aerobic conditions, increasing only upon exposure to air.

This evidence provides useful insight into the evolution of off-flavors resulting from disproportionate ethyl−ester content in apple fruit stored under certain ultralow O_2 CA conditions. Hypoxic CA conditions, while responsible for the increased biosynthesis of ethanol, may actually limit the formation of ethyl esters while these storage conditions are maintained. More importantly, the procedure by which the fruit are exposed to air following CA storage may have a greater effect on the final flavor profile of post-CA storage fruit.

Specific Effects of Anaerobiosis on Aroma Synthesis. Because of the effects of anaerobiosis on ester synthesis, it is important to discuss which specific steps may be inhibited by oxygen deprivation. Synthesis of various alcohols and esters responsible for flavor may be directly inhibited at many steps by anoxia. Moreover, the effects of anoxia on many processes, including ATP synthesis and oxidation of various cofactors during respiration, would indirectly affect aroma volatile synthesis. In relation to substrate production for aroma volatile synthesis, the lipoxygenase (*28*) and β-oxidation (*29*) pathways are oxygen-dependent. The proposed mechanism for α-oxidation is oxygen-dependent (*30*). The synthesis of acylated CoA, which is catalyzed by acyl CoA synthetase, is ATP-dependent (*31*). Nonglycolytic ATP production is oxygen-dependent (*21*). Plant aroma ester biosynthesis (*8*, *32*−*34*) and β-oxidation (*29*) require acyl CoA as an initial substrate. The decarboxylation of 2-keto acids in the formation of isovaleric acid (*35*) and the oxidation of primary alcohols to aldehydes (*36*) are reportedly NAD^+-dependent. The biosynthesis of this cofactor is largely impeded by anaerobiosis (*21*).

Because of the extensive biochemical effects, deductions based on the formation of constituent compounds may be errant. However, by treating tissue disks with 1-pentanol, some deductions may be made. The oxidation of 1-pentanol to pentanal, a reaction catalyzed by alcohol dehydrogenase (ADH) with NAD^+ as a cofactor (*36*), may be impeded by anoxia. The specific reasons for the inhibition of pentyl propionoate and pentyl pentanoate production are not as conclusive under the conditions of these tests. Use of specific inhibitors or sequestering portions or individual steps in this pathway, such as β-oxidation, could yield concrete results. Because these esters

are oxidized products of the feed alcohol, the inhibition may occur at the step catalyzed by ADH, the addition of CoA, the esterification reaction catalyzed by AAT, and/or β-oxidation, in the case of pentyl propionate. For similar reasons, the inhibition of pentyl acetate, pentyl butyrate, and pentyl hexanoate biosyntheses under anoxic conditions could occur at many different steps within the system.

Some steps are inhibited by hypoxia in fruit. Brackmann et al. (*18*) reported that low fatty acid synthesis/degradation may curtail ester production under these conditions. The effects of anoxia on the activity of AAT in higher plants remains to be elucidated. Yeast (*Saccharomyces* spp.) AAT activity (*37*) and gene expression (*38*) are inhibited by oxygen. In the present study, apple fruit AAT appears to be affected by anoxia. Biosynthesis of all of the esters detected increased dramatically after anoxic treatments were returned to air. However, nonester volatiles did not increase. Again, this may be due to a sudden increase in substrate for this reaction that rapid esterification after the exposure to air may have masked.

A decrease in the reduction of aldehydes, as indicated by a build-up of aldehyde precursor, has also been suggested to result in the lack of ester production resulting from long-term CA storage (*7*). As already mentioned, ethanol production rose more rapidly under anoxic conditions providing ample substrate for ethyl−ester biosynthesis. However, this was not true with the other alcohols reported in this study. Partially purified extracts of apple fruit ADH have a higher affinity for acetaldehyde than larger straight and branched chain aldehydes (*36*). High levels of acetaldehyde may out-compete butanal and hexanal for reduction. Additionally, substrate availability for these compounds, which are derived from different pathways (acetaldehyde from glycolysis and butanal and hexanal from lipoxygenase and/or β-oxidation), differs. Chervin et al. (*39*) report that neither PDC nor ADH are the limiting factors of ethanol production in pear fruit stored under hypoxic conditions but, instead, substrate availability. Interestingly, Nanos et al. (*40*) suggest that different ADH genes may be responsible for the synthesis of ADH isozymes in air- and hypoxia-treated pear fruits.

The level of aroma volatile biosynthesis is dependent upon tissue type, and in general, with the exception of ethanol, anoxia greatly reduces aroma volatile synthesis. Poststorage apple fruit skin synthesized pentanal and pentyl acetate at a higher rate and pentyl propionate, pentyl butyrate, and pentyl hexanoate at a significantly higher rate than the other two tissue types. Additionally, pentanal and pentyl acetate, each only one metabolic step from 1-pentanol, were produced at a higher rate than any of the other 1-pentanol-derived compounds. As suggested by previous investigations as well as this investigation, skin tissue may have increased amounts of constituent fatty acids, as well as greater capacity to oxidize a primary alcohol feed compound, and more capacity to synthesize aroma esters.

In general, anoxia adversely affected aroma volatile synthesis. Biosynthesis of pentanal as well as all of the reported esters derived from 1-pentanol may have been impeded by anoxia at many steps, both directly and indirectly. These data suggest that oxidation of the feed compound is adversely affected by anoxia. Additionally, ester biosynthesis, lipoxygenase activity, and β-oxidation appear to be curtailed by anoxia. Dramatic increases in the biosyntheses of all esters, both constituent and 1-pentanol-derived, upon exposure to air by tissue disks kept under anoxic conditions, may indicate inhibition of AAT by anoxia. Biosyntheses of constituent primary alcohols larger than ethanol appeared to be unaffected by anoxia, showing a downward trend in biosynthesis in both aerobic and anoxic treatments. However, ethanol biosynthesis increased at a greater rate in fruit held under anoxic conditions. Ethanol-derived ester biosyntheses remained low in samples held under anoxic conditions but rose dramatically when samples were exposed to air. This indicates that anoxic conditions actually inhibit ethyl−ester synthesis while promoting the production of its ethanol substrate. The nonconformity in the production of ethanol and other primary alcohols under anoxic conditions indicates changes in the availability of substrate for these pathways or perhaps different expression of ADH isozymes, as previously suggested in pear fruit.

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